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Doxepin and diphenhydramine increased non-rapid eye movement sleep through blockade of histamine H_1 receptors

Yi-Qun Wang [a,1], Yohko Takata [b,c,1], Rui Li [a], Ze Zhang [a,d], Meng-Qi Zhang [a,e], Yoshihiro Urade [b,c], Wei-Min Qu [a,d,*], Zhi-Li Huang [a,d,e,*]

[a] Department of Pharmacology and Shanghai Key Laboratory of Bioactive Small Molecules, School of Basic Medical Sciences, Fudan University, Shanghai, China
[b] Department of Molecular Behavioral Biology, Osaka Bioscience Institute, Osaka, Japan
[c] International Institute for Integrative Sleep Medicine (WPI-IIIS), University of Tsukuba, Tsukuba, Japan
[d] The Institutes of Brain Science, Shanghai Medical College, Fudan University, Shanghai, China
[e] State Key Laboratory of Medical Neurobiology, School of Basic Medical Sciences, Fudan University, Shanghai, China

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ABSTRACT

Histaminergic neurons have been reported to play an important role in the regulation of sleep–wake behavior through the histamine H_1 receptor (R, H_1R). First generation H_1R antagonists, such as doxepin and diphenhydramine, produce drowsiness in humans, and are occasionally used to treat insomnia. However, if H_1R antagonists function via physically blocking the H_1R remains unclear. In the current study, we used H_1R knockout (KO) mice to investigate if the sleep-promoting effects of doxepin and diphenhydramine are dependent on blockade of the H_1R. When doxepin was administered, non-rapid eye movement (NREM) sleep in wild type (WT) mice increased for 4 h, with an increase in the numbers of NREM sleep bouts of 256–512 s and 512–1024 s. These effects were not observed in the H_1R KO mice. Furthermore, diphenhydramine increased NREM sleep for 6 h in WT, and not in the H_1R KO mice after the injection. These results indicate that both doxepin at 15 mg/kg and diphenhydramine at 10 mg/kg induce NREM sleep through blockade of H_1R.

1. Introduction

Histaminergic neurons are primarily located in the tuberomammillary nucleus and adjacent regions of the brain (Inagaki et al., 1988; Lin et al., 1988; Rodenbeck et al., 2003; Thakkar, 2011). Histamine exerts its effects through four receptors (H_1, H_2, H_3 and H_4), which are primarily found in the brain (Bouthenet et al., 1988; Martinez-Mir et al., 1990). Previous research has demonstrated potentiating brain histaminergic activity induces wakefulness and reduces sleep via regulation of the H_1 receptor (H_1R) (Lin et al., 1988; Monti et al., 1986; Tokunaga et al., 2009). The H_1R is a typical G-protein coupled metabotropic receptor with seven putative transmembrane domains (Yamashita et al., 1991). This receptor can be found in the regions including basal forebrain, locus coeruleus, raphe nuclei, mesopontine tegmentum and the thalamus which regulate sleep and wakefulness in the brain (Palacios et al., 1981). Thus, the H_1R is prevalent in key areas of the brain that are required to be activated for wakefulness to occur. H_1Rs antagonism has been demonstrated to reduce wakefulness (Ikeda-Sagara et al., 2012; Shigemoto et al., 2004; Tokunaga et al., 2007; Unno et al., 2012).

The sleep-promoting effects of first generation H_1R antagonists were first reported in the 1930s, and drugs that increase sleep by blocking the H_1R have existed for about 80 years (Yanai et al., 1992). Most of these drugs, such as diphenhydramine, are the key constituents of over-the-counter (OTC) sleep aids (Krystal, 2009; Schutte-Rodin et al., 2008). Another first generation H_1R antagonist, doxepin, is a tricyclic antidepressant. It has significant anti-histaminergic activity and is sometimes used to treat insomnia (Hajak et al., 2001; Weber et al., 2010). Although these H_1R antagonists have been used for many years in treating insomnia, the underlying mechanism of these drugs is poorly understood. In addition, these agents also bind to other non-histaminergic receptors, producing other effects and can influence the sleep-promoting effects of these drugs. For example, α_2 adrenergic receptor inhibition, serotonin (5-HT) reuptake inhibition, and 5-HT_{2c} receptor antagonism will lead to wakefulness and counteract the sedative effects of H_1R blockade (Monti, 2010; Prast et al.,

Abbreviations: 5-HT, serotonin; EEG, electroencephalogram; EMG, electromyogram; H_1R, histamine H_1 receptor; KO, knockout; NREM, non-rapid eye movement; OTC, over-the-counter; REM, rapid eye movement; WT, wild type

* Corresponding authors at: Department of Pharmacology, School of Basic Medical Sciences, Fudan University, Shanghai 200032, China. Tel.: +86 21 54237043; fax: +86 21 54237103.
E-mail addresses: quweimin@fudan.edu.cn (W.-M. Qu), huangzl@fudan.edu.cn (Z.-L. Huang).

[1] YQW and YT contributed equally to this work.

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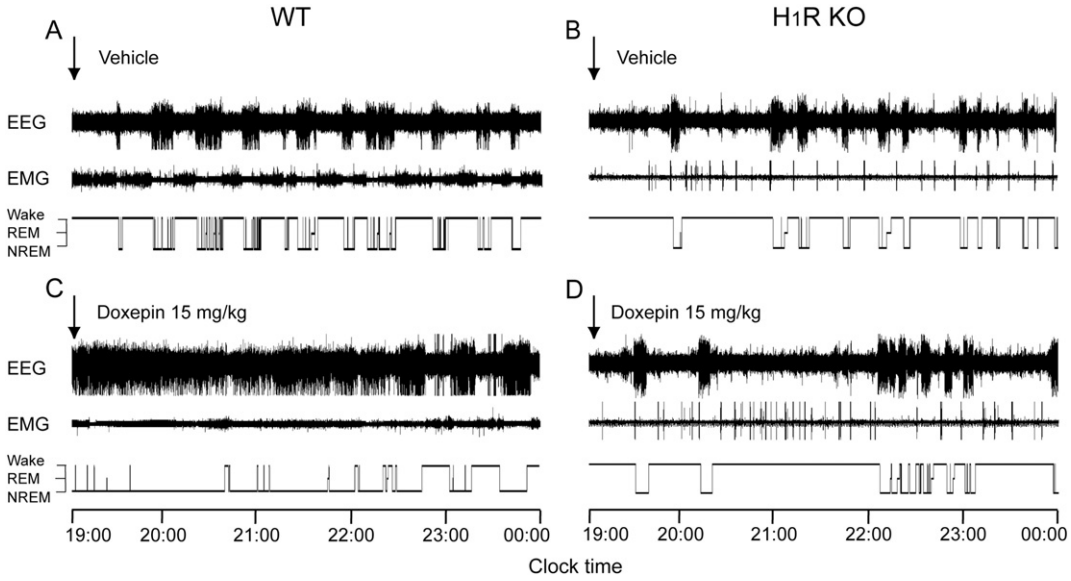

Fig. 1. Typical examples of EEG, EMG, and hypnograms after administration of vehicle or doxepin (15 mg/kg, p.o.) in a WT (A, C) and an H_1R KO mouse (B, D). Arrows in A, B, C and D indicate the time of administration.

1993, 1994; Wafford and Ebert, 2008). Therefore, in this study, we explored the mechanisms underlying the sleep-promoting effects of these two H_1R antagonists.

In the present study, we used H_1R knockout (KO) mice to elucidate the mechanisms by which doxepin and diphenhydramine mediate sleep-promoting effects. Previously, H_1R KO mice (Huang et al., 2006; Inoue et al., 1996) have been used as a unique tool to investigate the role of H_1R in the promotion of wakefulness and/or sleep inhibition induced by various drugs (Huang et al., 2006). In this study, we demonstrate both doxepin and diphenhydramine promoted non-rapid eye movement (non-REM, NREM) sleep in wild type (WT) but not H_1R KO mice.

2. Materials and methods

2.1. Animals

Male H_1R KO mice (Huang et al., 2001; Inoue et al., 1996; Schutte-Rodin et al., 2008) and their littermate controls of the inbred C57BL/6J strain were generated from heterozygotes, and maintained at Oriental Bioservice (Kyoto, Japan). The animals used in the current experiments weighed 20–26 g (11–13 weeks old). The animals were housed in individual cages at a constant temperature of 22 ± 0.5 °C with a relative humidity level of 60 ± 2% on an automatically controlled 12-h light/12-h dark cycle (lights on at 07:00 h). Animals had free access to food and water. The experimental protocols were approved by the Animal Research Committee of Osaka Bioscience Institute. All efforts were made to minimize the number of animals used and any pain or discomfort experienced by the subjects.

2.2. Polygraphic recordings and vigilance state analysis

Under pentobarbital anesthesia (50 mg/kg, i.p.), mice were implanted with electrodes for polysomnographic electroencephalogram (EEG) and electromyogram (EMG) recordings. Two stainless steels screws (1 mm in diameter) were inserted through the skull (antero-posterior, +1.0 mm; left–right, −1.5 mm from bregma or lambda) according to the mouse brain atlas (Franklin and Paxinos, 1997). These screws served as EEG electrodes. Two Teflon-coated, insulated stainless steel wires were placed bilaterally into both trapezius muscles, and these served as the EMG electrodes. All electrodes were attached to a microconnector and fixed onto the skull by dental cement.

After a 10-day recovery period, each mouse was transferred to a sound-proof recording chamber, and connected to an EEG/EMG recording cable for a 4-day period of habituation to the experimental environment. Polygraphic recordings were recorded for 48 h, in freely moving mice.

Cortical EEG and EMG signals were amplified, filtered (EEG, 0.5–30 Hz; EMG, 20–200 Hz), digitized at a sampling rate of 128 Hz and recorded by using Sleepsign (Kissei Comtec, Nagano, Japan) as described earlier (Huang et al., 2001, 2005; Liu et al., 2012; Qu et al., 2010, 2012). When completed, polygraphic recordings were automatically scored off-line at 4-s epochs as wakefulness, rapid eye movement (REM), and non-rapid eye movement (NREM) sleep, by SLEEPSIGN, according to standard criteria (Huang et al., 2001, 2005; Qu et al., 2010; Wang et al., 2012; Yan et al., 2011). Finally, defined sleep–wake stages were examined visually, and corrected when necessary.

2.3. Pharmacological treatments

Doxepin (Sigma-Aldrich) were dissolved in water and administered per os (p.o.), before darkness onset, at 19:00 h. Diphenhydramine (Sigma-Aldrich) was dissolved in sterile saline and administered intraperitoneally, before darkness onset, at 19:00 h.

2.4. Statistical analysis

All results are expressed as means ± SEM. For vigilance studies, amounts of sleep–wake states are expressed in minutes. For time-course changes, the hourly amounts of each stage in mice, treated with drug or vehicle, were analyzed with repeated measures ANOVA followed by *post hoc* Fisher's Probable Least-Squares Difference (PLSD) test. The total amount of sleep and wakefulness, number of episodes, mean duration, number of stage transitions, number of bouts and the EEG power density between vehicle and treatment groups were analyzed using the paired, two-tailed Student's *t*-test. In all cases, $p < 0.05$ was taken as the level of significance.

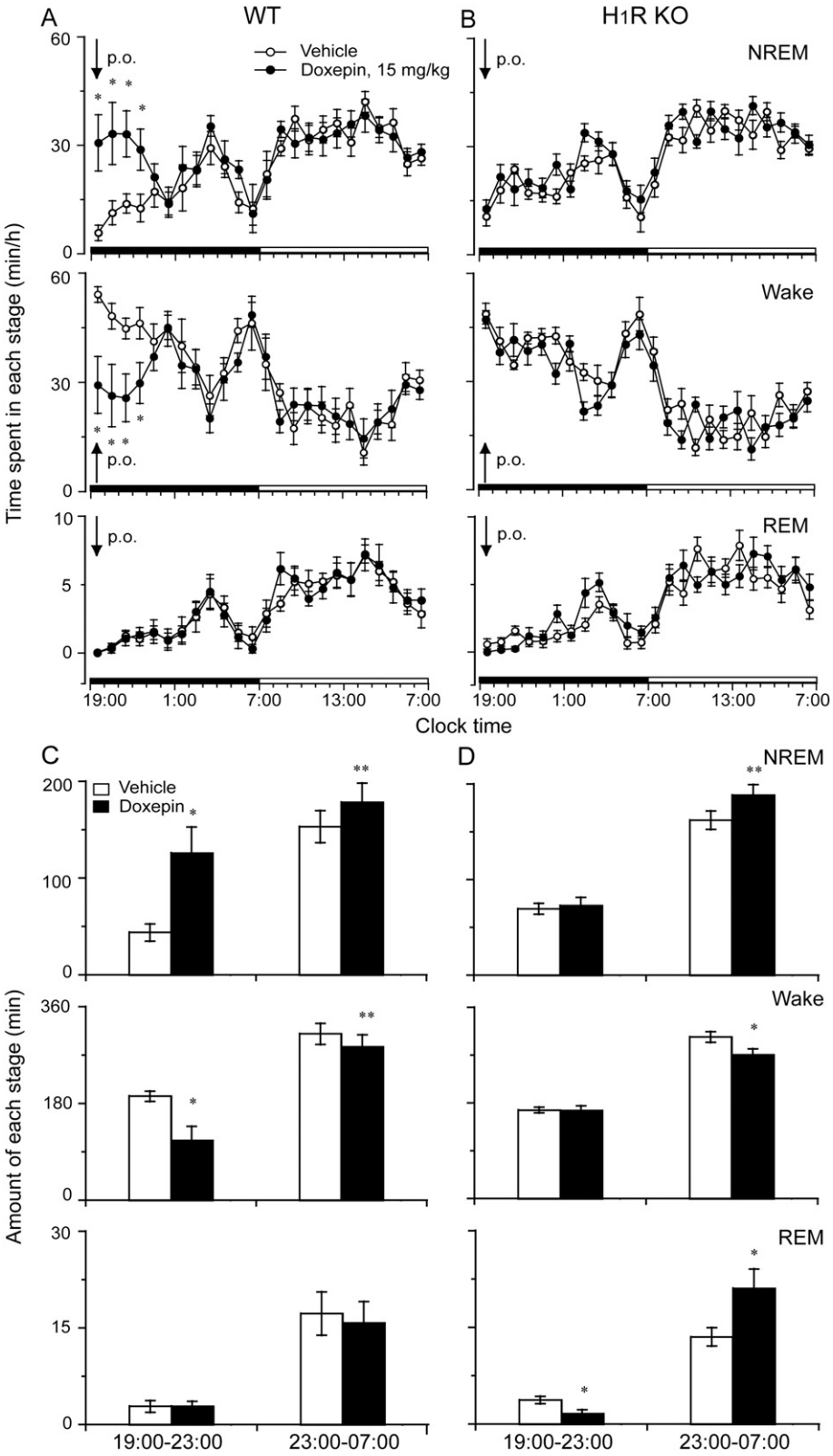

Fig. 2. Effects of doxepin on sleep–wake profiles in WT and H_1R KO mice. Time course changes in wakefulness, NREM and REM sleep in WT (A) and H_1R KO (B) mice treated with doxepin. Each circle represents the hourly mean amount of each stage. Open and closed circles stand for the profiles of vehicle and doxepin treatments, respectively. The horizontal filled and open bars on the χ-axes indicate the 12-h dark and 12-h light periods, respectively. Values are the means ± SEM (n = 7–8). $^{*}P < 0.05$, indicates significant differences of experimental groups compared to vehicle groups, as assessed by repeated measures ANOVA, followed by the PLSD test. Total time spent in wakefulness, NREM, and REM sleep, during the 4-h period (19:00-23:00) after administration of vehicle or doxepin, and the subsequent 8-h period (23:00-07:00), in WT (C) and H_1R KO (D) mice. Open and filled bars show the profiles of vehicle and doxepin treatments, respectively. Values are the means ± SEM (n = 7–8). $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate significantly differences between the doxepin-treated group and its corresponding vehicle control group, as assessed by two-tailed paired Student's *t-test*.

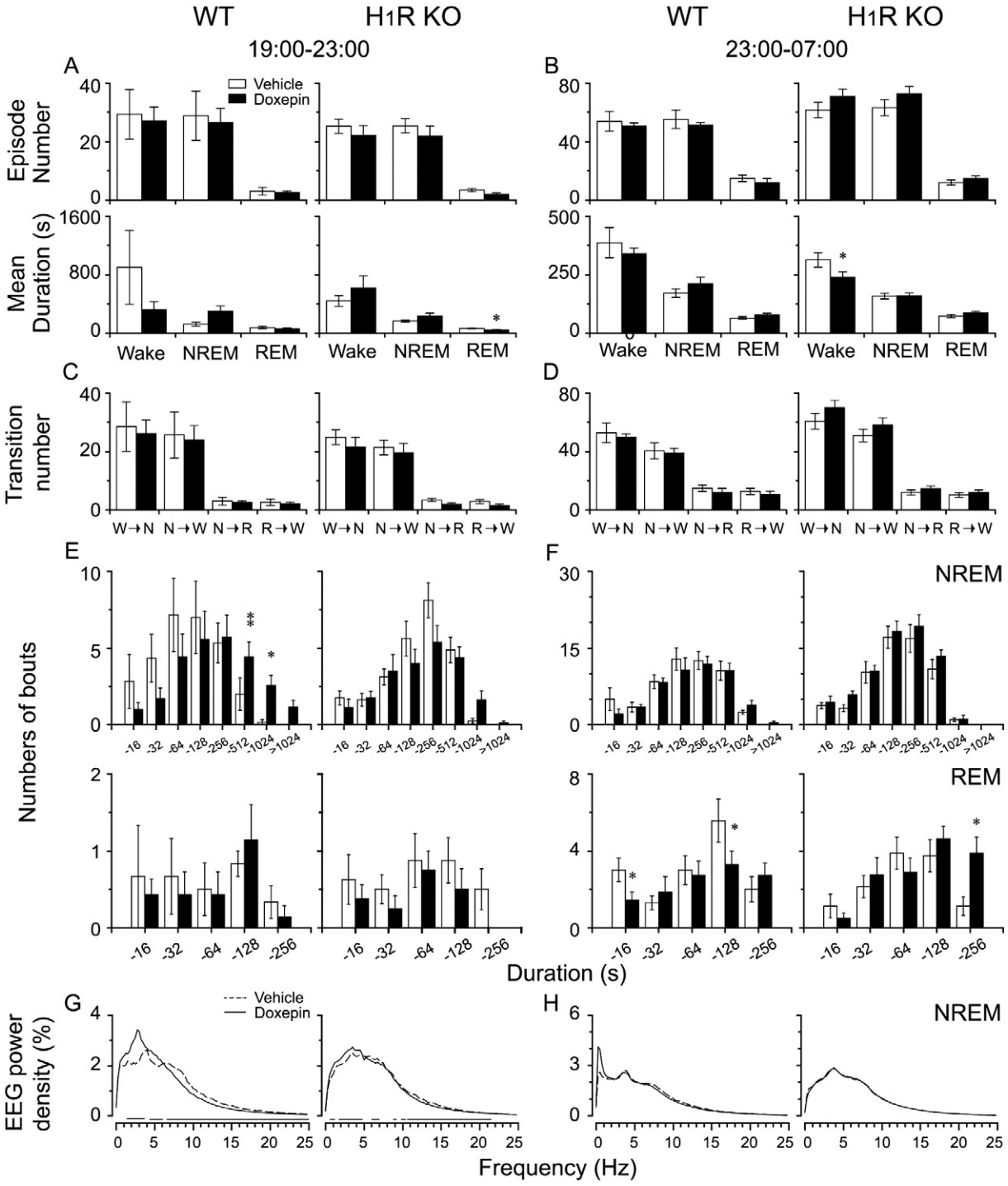

Fig. 3. Episode numbers, and mean durations (A, B), stage transition (C, D), and numbers of NREM and REM sleep bouts (E, F), during the 4-h period after administration of vehicle or doxepin, and from 23:00 to 07:00 h, in WT and H_1R KO mice. Open and filled bars show the profiles for the vehicle and doxepin treatments, respectively. Values are the means ± SEM (n = 7–8). $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate significant differences between the doxepin-treated group and its corresponding vehicle control group, as assessed by two-tailed paired Student's *t-test*. EEG power density curves during the 4-h period after administration of vehicle or doxepin (G), and from 23:00 to 07:00 h (H), in WT and H_1R KO mice. The power of each 0.25-Hz bin was averaged and normalized by calculating the percentage of each bin from the total power (0–24.75 Hz). The horizontal bars indicate where there is a statistical difference ($P < 0.05$) between vehicle and doxepin treatments. W, wakefulness, N, NREM sleep, R, REM sleep.

3. Results

3.1. Doxepin did not increase NREM sleep in H_1R KO mice

The typical examples of EEG, EMG and hypnograms, after WT and H_1R KO mouse were treated with vehicle or doxepin are shown in Fig. 1. Compared to vehicle controls, doxepin increased NREM sleep in the WT, but not in the H_1R KO mouse during the period of 19:00–23:00 h. Doxepin at 15 mg/kg markedly increased NREM sleep ($F_{1,132} = 4.884$, $p < 0.05$) during the dark period, compared to the vehicle control, beginning the first hour after doxepin administration at the onset of darkness in WT mice (Fig. 2A). This increase in NREM sleep was maintained for 4 h after administration. Furthermore, this increase in NREM sleep was not observed in H_1R KO mice, when compared to the vehicle control (Fig. 2B). The increase in NREM sleep was concomitant with the decrease in wakefulness in WT

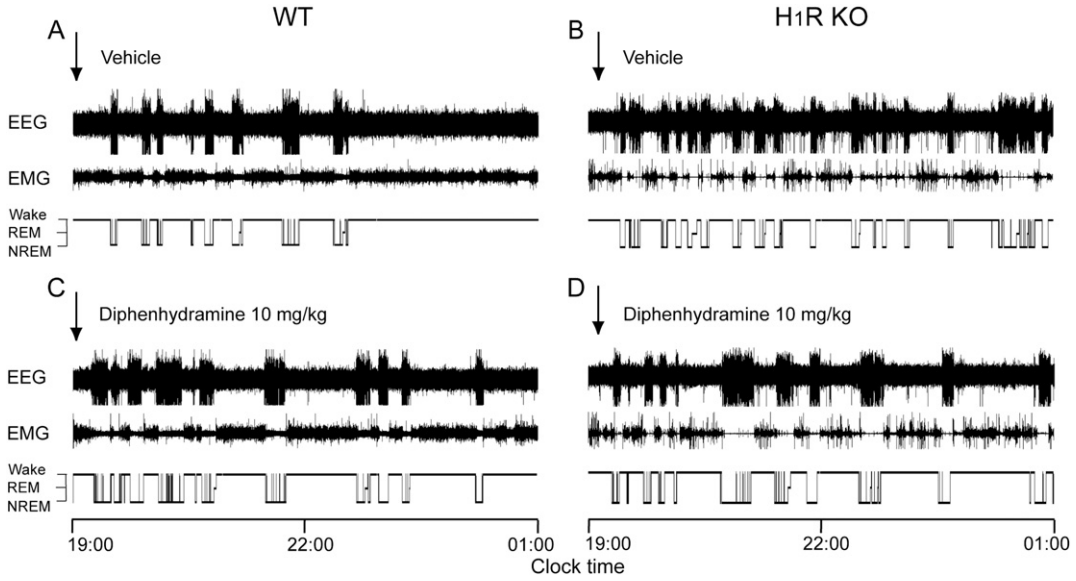

Fig. 4. Typical examples of EEG, EMG, and hypnograms after administration of vehicle or diphenhydramine (10 mg/kg, i.p.), in a WT (A, C) and H_1R KO mouse (B, D). Arrows in A, B, C, and D indicate the time of injection.

mice, after doxepin treatment (Fig. 2A). Finally, the sleep architecture during the subsequent dark and light periods in both WT and H_1R KO mice, was not disrupted (Fig. 2A and B).

The total time spent in wakefulness, NREM, and REM sleep was calculated for the 4-h period after doxepin administration, as well as the subsequent 23:00–7:00 h period. The total amount of NREM sleep, in WT mice, increased during both the 19:00–23:00 h and 23:00–7:00 h by 1.9-fold ($p < 0.05$) and 16.4% ($p < 0.01$), respectively, but only increased slightly (16.2% with a $p < 0.01$) in H_1R KO mice during the 23:00–7:00 h (Fig. 2C and D). The concomitant decreases in the total amount of wakefulness, in WT mice, during 19:00–23:00 h, and 23:00–7:00 h were 42.6% ($p < 0.05$) and 7.7% ($p < 0.01$), respectively, but only decreased in the H_1R KO mice during the 23:00–7:00 h period by 11.1% ($p < 0.05$) (Fig. 2C and D). The amount of REM sleep observed in WT mice was similar between the vehicle and doxepin groups (Fig. 2C). Doxepin decreased REM sleep during the 19:00–23:00 h by 56.6% ($p < 0.05$), but subsequently increased REM sleep between 23:00 and 7:00 h by 35.7% ($p < 0.05$) in H_1R KO mice (Fig. 2C and D). These results indicate that doxepin at 15 mg/kg increased NREM sleep for the 4-h period after administration via the H_1R.

3.2. Doxepin did not increase the number of NREM sleep bouts in H_1R KO mice

To better understand the changes in the sleep architecture caused by doxepin, the mean duration of the three vigilance stages, transition numbers, the numbers of NREM and REM sleep bouts, and the EEG power density of NREM sleep were determined (Fig. 3). During the 4-h period immediately following administration, doxepin decreased the mean duration of REM sleep by 30.1% ($p < 0.05$) in H_1R KO mice, but not in WT mice (Fig. 3A). During the 23:00–7:00 h, doxepin decreased the mean duration of wakefulness by 23.7% ($p < 0.05$) in H_1R KO mice, but not in WT mice (Fig. 3B). The stage transitions between sleep and wakefulness were almost identical between vehicle control and the doxepin-treated group in both WT and H_1R KO mice (Fig. 3C and D). Spectral analysis of EEG power density indicated doxepin increased the EEG power density of NREM sleep in the frequency range of 1.25–3.75 Hz and 4.25–6 Hz, and decreased in the frequency range of 6.75–25 Hz in WT mice, during the 19:00–23:00 h (Fig. 3G). During the same time frame EEG power density increased in the 0.5–1.25 Hz and 1.75–4.75 Hz ranges and decreased in the 6–7 Hz, 8.75–9.25 Hz, 9.75–10.25 Hz, and 10.5–21.5 Hz ranges in the H_1R KO mice (Fig. 3G). During the subsequent period, neither WT nor H_1R KO mice demonstrated any significant change in EEG power density of NREM sleep in the frequency range of 0–24.75 Hz, after doxepin administration (Fig. 3H). For WT and H_1R KO mice in REM sleep, the EEG power density was nearly identical across a 0–24.75 Hz frequency range, after doxepin treatment (data not shown). These findings indicate that the effects of doxepin on stage-specific EEG activity are not dependent on the H_1R.

The numbers of NREM sleep bouts of 256–512 s and 512–1024 s were increased in WT mice, but remained unchanged in the H_1R KO mice, after doxepin administration, across the 19:00–23:00 h (Fig. 3E). During the 23:00–7:00 h, doxepin significantly decreased the number of 0–16 s REM sleep bouts (52.4%, $p < 0.05$) and 64–128 s REM bouts (41.0%, $p < 0.05$) in WT mice (Fig. 3F). In H_1R KO mice, the treatment increased the number of 128–256 s REM bouts by 2.4-fold ($p < 0.05$) (Fig. 3F). These results suggested that doxepin maintained NREM sleep through its activity on H_1R.

3.3. Diphenhydramine did not increase NREM sleep in H_1R KO mice

The effects of diphenhydramine (10 mg/kg), administrated via i.p. injections at 19:00 h were also analyzed. Typical examples of EEG, EMG and hypnograms, after the administration of vehicle or diphenhydramine, in a WT and H_1R KO mouse, are shown in Fig. 4. Diphenhydramine increased NREM sleep in WT mice between 19:00-20:00 h, compared to the control group (Fig. 5A). Moreover, this increase in NREM sleep was not observed in H_1R KO mice (Fig. 5B). The changes in NREM sleep were concomitant with the decreases in wakefulness in WT mice (Fig. 5A). When the total amount of NREM sleep was calculated, diphenhydramine increased NREM sleep between 19:00-1:00 h by 76.6% ($p < 0.01$) in WT mice (Fig. 5C). Diphenhydramine also increased NREM sleep in H_1R KO mice, but this change was not statistically significant (Fig. 5D). The total amount of wakefulness between 19:00-1:00 h decreased by 13.1% ($p < 0.01$) in the WT but not in the H_1R KO mice, after diphenhydramine injection (Fig. 5C and D). No difference in the amount of REM sleep was observed between the diphenhydramine and vehicle groups in either the WT or H_1R KO mice. These findings indicate that diphenhydramine decreased wakefulness also via blockade of H_1R.

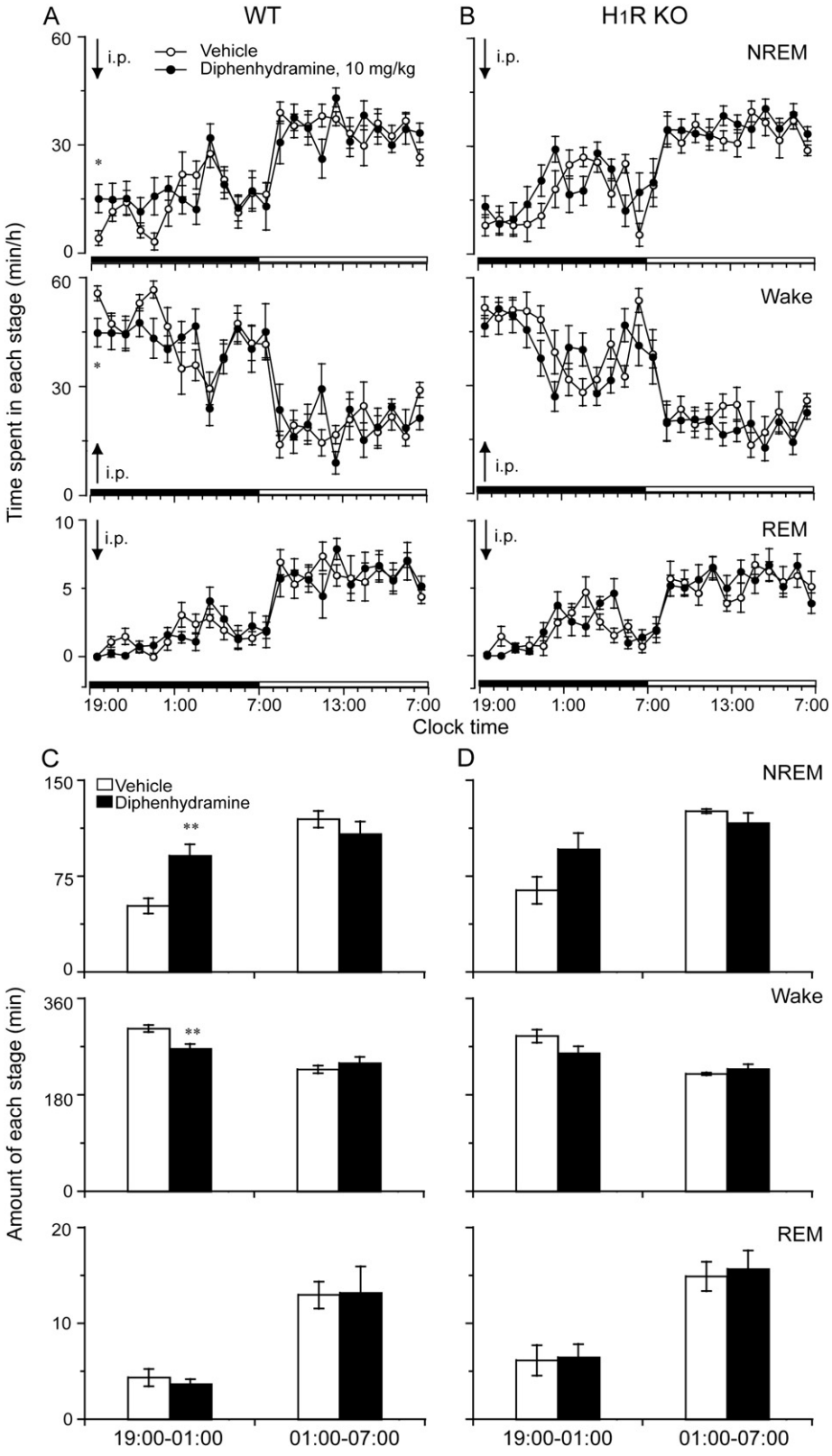

Fig. 5. Effects of diphenhydramine on sleep–wake profiles in WT and H_1R KO mice. Time course changes in wakefulness, NREM, and REM sleep in WT (A) and H_1R KO (B) mice treated with diphenhydramine. Each circle represents the hourly mean amount of each stage. Open and closed circles stand for the profiles of vehicle and diphenhydramine treatments, respectively. The horizontal filled and open bars on the χ-axes indicate the 12-h dark and 12-h light periods, respectively. Values are the means ± SEM (n = 7). $^{*}P < 0.05$ indicates a significant difference between the diphenhydramine-treated group and the vehicle group, as assessed by repeated measures ANOVA, followed by the PLSD test. Total time spent in wakefulness, NREM, and REM sleep during the 6-h period (19:00-01:00) after administration of vehicle or diphenhydramine and the subsequent 6-h period (01:00-07:00), in WT (C) and H_1R KO (D) mice. Open and filled bars show the profiles of vehicle and diphenhydramine treatments, respectively. Values are the means ± SEM (n = 7). $^{**}P < 0.01$ indicates significant difference between the diphenhydramine-treated group and its corresponding vehicle control group, as assessed by two-tailed paired Student's *t-test*.

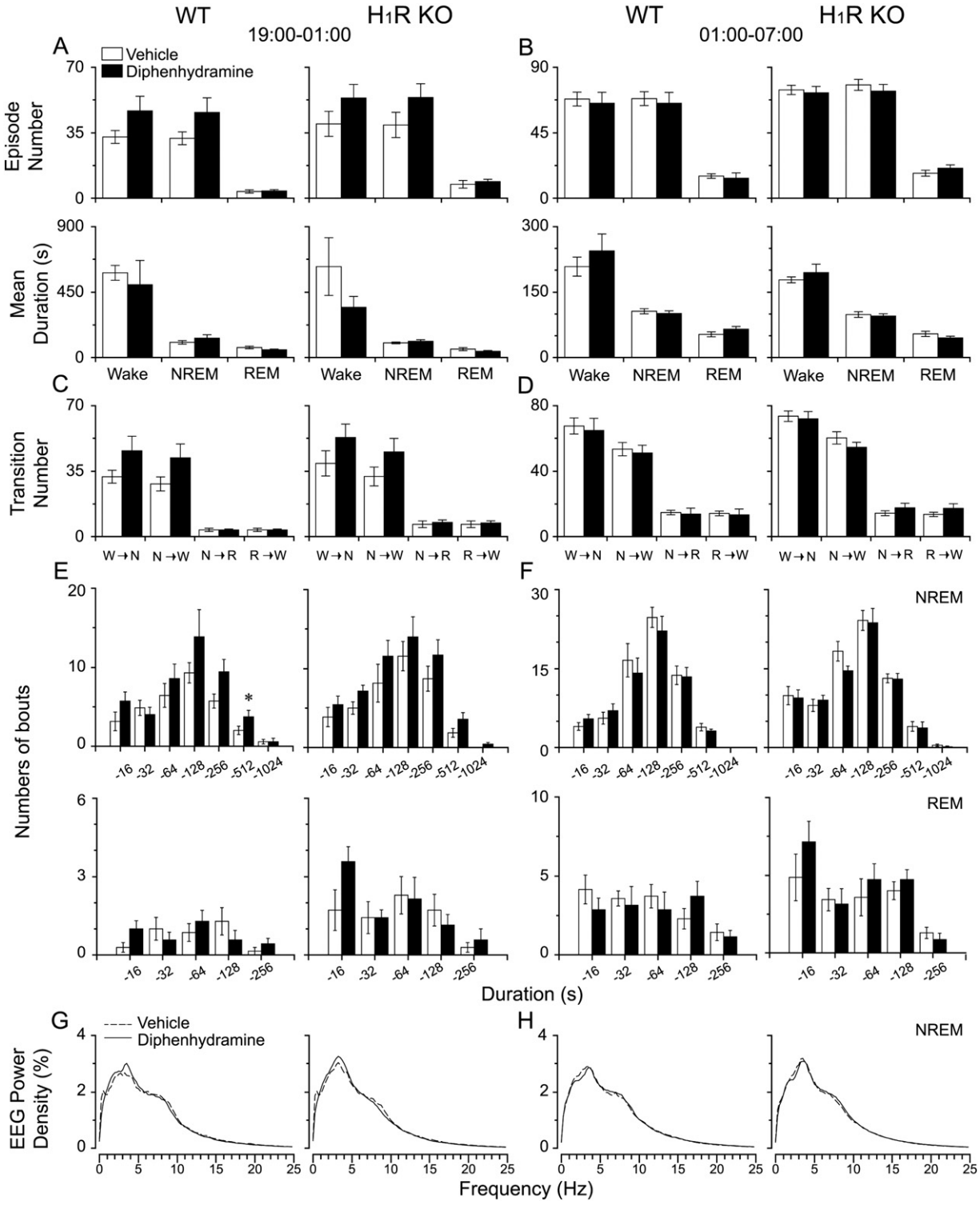

Fig. 6. Episode numbers, and mean durations (A, B), stage transition (C, D) and numbers of NREM and REM sleep bouts (E, F), during the 6-h period after injections of vehicle or diphenhydramine and from 01:00 to 07:00 h, in WT and H_1R KO mice. Open and filled bars show the profiles for the vehicle and diphenhydramine treatments, respectively. Values are the means ± SEM (n = 7). *$P < 0.05$ indicates significant differences from the corresponding vehicle control group as assessed by two-tailed paired Student's *t-test*. EEG power density curves during the 6-h period after administration of vehicle or diphenhydramine (G), and the subsequent 6-h period (H), in WT and H_1R KO mice. The power of each 0.25-Hz bin was averaged and normalized by calculating the percentage of each bin from the total power (0–24.75 Hz). W, wakefulness, N, NREM sleep, R, REM sleep.

3.4. Diphenhydramine did not increase the number of NREM sleep bouts in H_1R KO mice

The episode numbers and mean duration of three vigilance stages, state transitions between the three vigilance stages, and the EEG power density of NREM sleep in the diphenhydramine treatment group were comparable to those seen in the vehicle control group, in both WT and H_1R KO mice, during the 19:00–01:00 h and 01:00–07:00 h time periods (Fig. 6).

Between 19:00-1:00 h, the number of NREM sleep bouts of 256–512 s increased after diphenhydramine injection, in WT mice,

however this effect was not observed in H_1R KO mice (Fig. 6E). These findings indicate that the effect of diphenhydramine for maintaining NREM sleep mainly occurs through H_1R blockade.

4. Discussion

Histaminergic neurons primarily located in the tuberomammillary nucleus and proximal areas within the posterior hypothalamus, and send large efferents to other brain regions (Haas and Panula, 2003). There is significant evidence which has demonstrated histamine is critical in regulating wakefulness. During the period of wakefulness, histaminergic neurons discharge tonically and specifically (Vanni-Mercier et al., 2003). These neurons, however, fully shout off their firing during REM sleep period (Thakkar, 2011). Furthermore, the central release of histamine exhibits circadian rhythms related to the change of wakefulness and sleep (Chu et al., 2004), and wakefulness is promoted by the inducement of histaminergic connections with some wake-promoting agents, such as thioperamide (Lin et al., 1990) or orexin (Huang et al., 2001), and with prostaglandin E_2 or an agonist of prostaglandin E_2 receptor subtype EP_4 (Huang et al., 2003). The H_1R is one of four subtypes of histamine receptors, which are widely distributed in the brain (Haas and Panula, 2003; Tokunaga et al., 2009). The use of H_1R antagonists can promote sleep in clinical settings. If the H_1R antagonists have non-selective pharmacological activity, it is most likely that the sleep-promoting effects are due to H_1R blockade (Krystal et al., 2013).

Doxepin is a tricyclic antidepressant, that targets a variety of different receptors, including the α_1 and α_2 adrenergic receptors, muscarinic M_1 receptor, 5-HT_2 receptor, norepinephrine transporter, 5-HT transporter, and the dopamine transporter, in addition to the H_1R (Krystal et al., 2013; Stahl, 2008). These receptors have all been shown to have a role in wakefulness and sleep regulation. For example, inhibition of the α_2 adrenergic receptor and the 5-HT_{2c} receptor can induce arousal. Moreover, H_1R antagonists produce the same sleep-promoting effects as α_1 adrenergic receptor antagonists, as well as is produced by inhibitors of 5-HT_{2A} receptors, which promote slow-wave sleep (Gyongyosi et al., 2008; Haas and Panula, 2003; Huang et al., 2005; Vanni-Mercier et al., 2003). Furthermore, Rodenbeck et al. reported that sleep-promoting effects of doxepin are regulated through modulation of the hypothalamic–pituitary–adrenal axis functions (Rodenbeck et al., 2003). Doxepin has been shown to be one of the most potent H_1R antagonists available, and the blockade of H_1R is its principal pharmacologic effect (Hajak et al., 2001). Previous studies have shown doxepin has more than seven times greater affinity for the H_1R than any other receptors it has been tested (Krystal et al., 2013; Stahl, 2008). Therefore, doxepin is highly selective for H_1R at low dosages, considering that under such conditions, it would minimally bind to other receptors (Goforth, 2009). Here we show that doxepin induced NREM sleep in WT mice but not in H_1R KO mice. This strongly indicates drowsiness, induced by doxepin, is totally depends on H_1R antagonism. These results are in accordance with the finding that low dosages of doxepin are selective for H_1R. The therapeutic effects of doxepin are specifically associated with H_1R.

Diphenhydramine is a key ingredient in OTC sleep aids. Numerous reports have shown it induces sedation and drowsiness. Some clinical studies have demonstrated 50 mg a day of diphenhydramine was an effective and safe treatment for patients with insomnia (Kudo and Kurihara, 1990; Rickels et al., 1983). In the present study, we found that diphenhydramine at 10 mg/kg is an appropriate dose to induce sleep in mice. Based on the US Food and Drug Administration guidelines for human equivalent dose calculation, a 10 mg/kg dose in mice is equivalent to 0.92 mg/kg dose in humans. For a human, with a 70 kg body weight, the dose would be 64.4 mg a day. Therefore, the dose of 10 mg/kg may be enough for investigating the effect of diphenhydramine in mice. It has been shown that diphenhydramine decreases sleep latency and has been used clinically in patients with insomnia (Tokunaga et al., 2007). In addition to H_1R antagonism, this first generation H_1R antagonist has been shown to interact with other receptors, including the 5-HT, norepinephrine, and acetylcholine receptors, as well as their transporters. In addition, it has been reported to bind to muscarinic receptors and has anti-cholinergic activities (Kubo et al., 1987; Orzechowski et al., 2005; Reuse, 1948). Furthermore, it can also inhibit various K^+ channels (Sato et al., 2005; Taglialatela et al., 2000) and activate intracellular Ca^{2+} mobilization (Burde et al., 1996). The concentration of Ca^{2+} in medial preoptic neurons can be increased by antihistamine agent-D-chlorpheniramine independent of histamine (Ikeda-Sagara et al., 2012; Unno et al., 2012). Therefore, the sedative effect of diphenhydramine could result from interactions with other receptors; however, our results indicated the sleep-promoting effects of diphenhydramine are primarily mediated through the H_1R.

Previous studies showed that the affinity (Ki in nM) of doxepin with recombinant human H_1R, expressed in Chinese Hamster Ovary cells, is 0.78 nM (Krystal et al., 2013). Our in vitro results are in accordance with these studies, in that doxepin had greater affinity to H_1R, demonstrating that its effects on insomnia are mediated solely via this receptor. Here we found that this drug induced a robust, but inconsistent sleep effect. In comparison, the sedative effect of diphenhydramine was weaker, but lasted longer, and was without significant impairment in the subsequent period of sleep. These attributes account for the more common use of diphenhydramine instead of doxepin, as the key constituent in OTC sleep aids.

5. Conclusion

These findings indicate that the H_1R is essential for the drowsiness effects observed after doxepin and diphenhydramine administration.

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